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Less Neutrophil Extracellular Trap Formation in Term Newborns than in Adults

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Neonates · Neutrophil extracellular traps · Innate immunity · Perinatal infection

Abstract
Background: Newborns are prone to infections, which are independent predictors of neonatal mortality and morbidity. Neutrophil extracellular traps (NETs) are structures composed of chromatin and antimicrobial molecules that capture and kill pathogens. NETs may play an important role in the innate immune system and, thus, might be associated with impaired neonatal immune function. ***Objectives:*** This study aimed to compare NET formation between term neonates and healthy adults. We additionally investigated the effects of gestational age, birth weight, mode of delivery, gender, and perinatal infections. ***Methods:*** We collected cord blood from 57 term infants (mean gestational age, 39.1 weeks) and 9 late preterm infants (35 weeks), and peripheral blood from 18 healthy adult donors. Neutrophils were isolated, and then NET formation was induced using three different stimulants: N-formylmethionine-leucyl-phenylalanine, phorbol 12-myristate 13-acetate (PMA), or lipopolysaccharide. NETs were immunohistochemically stained and analyzed with regard to NET percentage and NET area. ***Results:*** With all three stimuli, healthy term infants showed a lower NET percentage than the adult control group ($p < 0.0001$ each). The groups also differed in NET area, but the significance level was lower. Following PMA stimulation, we observed greater reductions in NET percentage and NET area in preterm than term infants. ***Conclusions:*** The lower NET formation observed in term infants compared to adults likely contributes to the reduced neonatal immune response. NET formation appeared to be even further decreased in late preterm neonates. There remains a need for further investigations of NET formation in more immature preterm infants.

Introduction

The developing neonatal immune system differs from the adult immune system, making newborns highly susceptible to bacterial infection. Each year, an estimated 1 million neonates die within the 1st postnatal week due to severe sepsis or infection [1]. The risk of early infections is inversely related to birth weight, degree of prematurity, and socioeconomic status [2]. A study examining

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3,800 newborns reported septic shock in 1.3% of infants, with death due to infections especially common among neonates with a birth weight <1,000 g [3].

Neutrophils are the most abundant white blood cells in the human circulation. During infection, neutrophils participate in immune defenses by forming a physical barrier to engulf pathogens and exposing them to high local concentrations of antimicrobials. Neutrophils also play a role in cell death via a recently described new mechanism in which neutrophils release decondensed chromatin, histone, and antibacterial molecules into the extracellular space [4, 5].

Various diseases involve genetic and secondary acquired defects of neutrophil function. NADPH oxidase is a major enzyme in oxidative burst, and its activation is linked to the activation of intracellular granular proteases (e.g., neutrophil elastase and myeloperoxidase) and to the production of neutrophil extracellular traps (NETs) [6, 7]. Deficiencies in NADPH oxidase are associated with higher susceptibility to bacterial and fungal infections. Acquired defects in neutrophil function have been detected in cases of sepsis and traumatic brain injury. Additionally, elderly people and patients suffering from ischemic stroke injury seem to show reduced NETs [8, 9].

Neonates also show quantitative and qualitative deficiencies in neutrophils. Among children with a birth weight <1,000 g, 38% showed a blood neutrophil count <1,000/μL, which persisted for the first 7 days of life in 43% of this group [10]. Neonates also show reduced expression of Toll-like receptor (TLR) 4, impaired neutrophil chemotaxis and transmigration, defective phagocytosis, and decreased oxidative burst activity. These defects in neutrophil migration and killing mechanisms make neonates particularly susceptible to sepsis [11–15].

Limited evidence suggests that neonates may also show reduced ability to form NETs. One study investigating term and preterm infants <30 weeks of gestational age reported that these infants were unable to form NETs 1 h after induction with lipopolysaccharide (LPS) or platelet-activating factor [16]. In contrast, in a very small cohort of 3 neonates, it was shown that neutrophils were equally potent in the formation of NETs as adult neutrophils after a longer LPS stimulation period (3 h) [17]. To increase our understanding of this issue, we investigated NET formation in a large cohort comparing particularly term neonates to healthy adults. Furthermore, the size of our cohort allowed to analyze how NET release was influenced by important neonatal factors, including birth weight, birth mode, gestational age, and perinatal infection.

Methods

Study Population

Term and preterm (gestational age <37 weeks) neonates were recruited between July 2014 and October 2015 at the University Medicine Greifswald in Germany. Gestational age was calculated from the 1st day of the last menstrual period and confirmed by clinical examination. Healthy adults served as controls. Exclusion criteria were severe congenital malformations, chromosomal aberrations, and lack of written consent. Perinatal infection or chorioamnionitis were not exclusion criteria.

Perinatal infection was defined based on clinical symptoms and C-reactive protein (CRP) serum concentrations >20 mg/L within 72 h postnatally. These infants were treated with intravenous antibiotics. Treatment duration depended on CRP levels (3–5 days) [18]. Newborns without these conditions were defined as noninfected.

Ethics Statement

The study protocol was approved by the Ethics Committee of the Medical Faculty of the University of Greifswald (registration code B133/12). All parents and adult donors directly provided written informed consent.

NET Assay

Venous umbilical cord blood was collected immediately after birth. Immune responses of neutrophils decrease the longer the cells remain unprocessed. Thus, each NET assay was started within 2 h after blood collection, necessitating precisely timed organization between the delivery room, department of neonatology, and laboratory.

Neutrophils were isolated from heparinized whole blood using standard Histopaque/Percoll gradient centrifugation. Cells were resuspended in Hanks' balanced salt solution and diluted to 5×10^4 cells/mL. The cell suspension was cultured in 24-well plates (1 mL/well) and allowed to rest for 30 min at 37°C with 5% CO_2. NET formation was induced with either N-formylmethionine-leucyl-phenylalanine (fMLP) (0.9 nmol/L), phorbol 12-myristate 13-acetate (PMA) (1.5 nmol/L), or LPS (1 μg/mL) for 2 h at 37°C with 5% CO_2. Unstimulated neutrophils were used as a negative control.

After NET induction, we added the non-cell-permeant DNA-binding dye SYTOX Green to the cells to detect extracellular DNA. For each sample, we acquired 10 fluorescent and phase-contrast images using a LEICA DBMI-4000b microscope. To calculate NET percentages and efficiency, we counted the total and NET-forming neutrophils, and measured the fluorescent area covered by each cell using Fiji software (version 1.46). NETs were defined as a fluorescent area ≥300 μm^2 [19] (Fig. 1).

This method enables NET analysis while excluding bias due to necrotic cells. Automatic analysis is limited since it does not enable differentiation between necrosis and NET formation. In our experiments, we also added annexin to clearly distinguish between NET formation, apoptosis, and necrosis. Overall (including all experiments), the percentage of apoptotic cells was 4.3% and the percentage of necrotic cells was <0.5%. Only NET formation changed during our experiments.

Statistical Analysis

All data were analyzed in an exploratory manner using GraphPad-PRISM 5.0 (GraphPad, Inc., San Diego, CA, USA). Normal distribution was assessed using the Kolmogorov-Smirnov test.

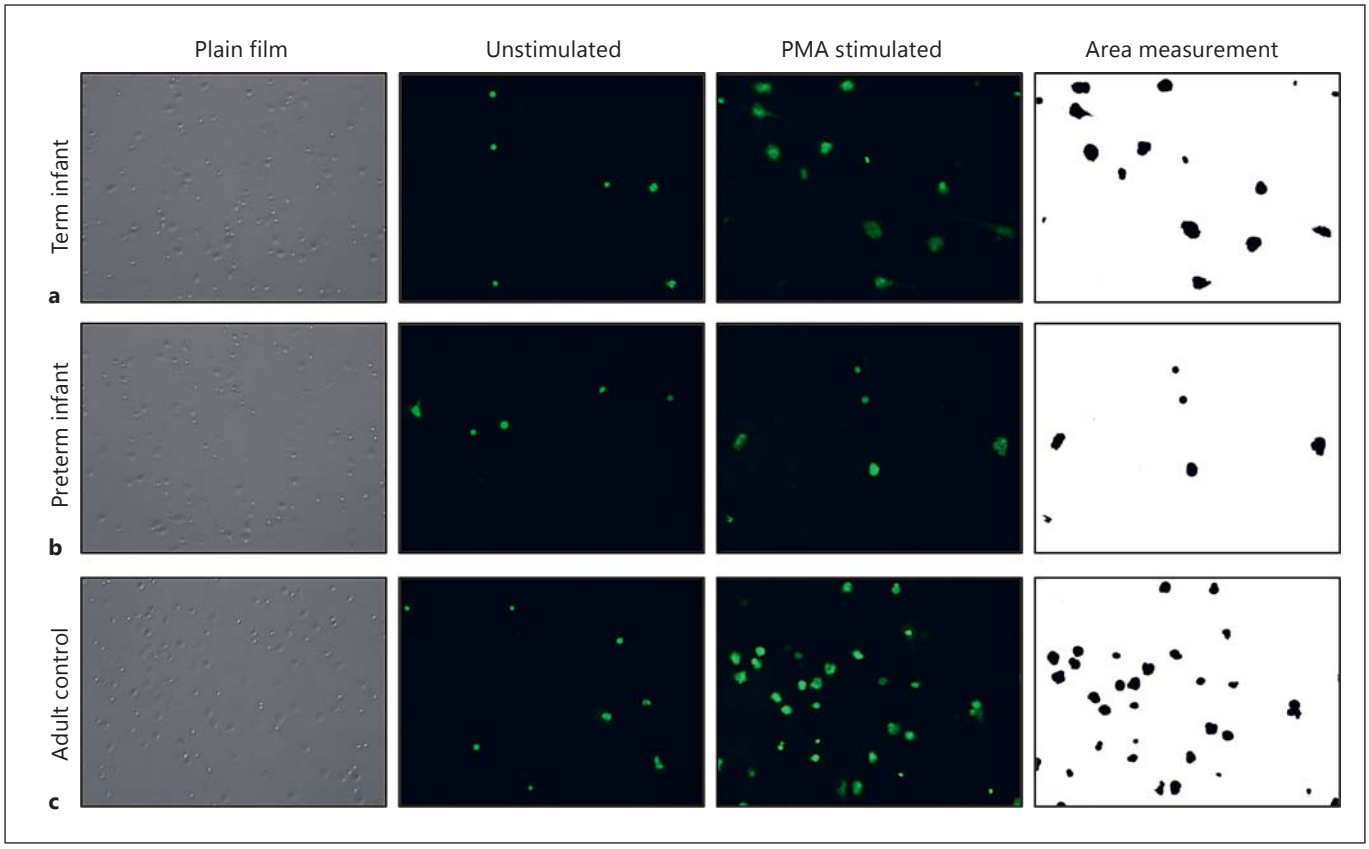

Fig. 1. Representative in vitro images of NET-forming neutrophils from a term infant (**a**), preterm infant (**b**), and adult control (**c**). Each row includes a plain film image, and SYTOX® Green-stained samples without stimulation and with phorbol 12-myristate 13-acetate (PMA) stimulation. The fourth column shows edited images of the PMA-stimulated samples from which the NET area was calculated using Fiji software. Images of SYTOX® Green-stained cells were taken using a Leica DMI 4000 B microscope with a 170-ms exposure time.

Table 1. Subject characteristics

	Term infants (n = 57)	Preterm infants (n = 9)	Adults (n = 18)
Age, years	n.a.	n.a.	26.7±7.5
Gestational age, weeks	39.03±1.0	35.04±1.3	n.a.
Birth weight, g	3,309.4±404.2	2,482.3±223.5	n.a.
Female	30 (53)	7 (78)	7 (39)
Male	27 (47)	2 (22)	11 (61)
Spontaneous delivery	18 (32)	3 (33)	n.a.
Infection	9 (16)	0	0

Means ± SD or n (%). n.a., not applicable.

Differences between populations were assessed using a two-tailed Student *t* test for normally distributed data. A value of $p \leq 0.05$ was considered to indicate significance. Means are given with standard deviations.

At the start of this project, the expected difference between newborns and adults or the distribution of data among newborns was not known. Thus, the results are presented as purely descriptive. However, a post hoc power analysis resulted in a power of 100% for comparing the percentage of NET formation between neonates and adults.

Results

Table 1 presents the characteristics of the patients and controls. Blood culture was negative in infants with perinatal infection.

Compared to the adult control group, both the term and preterm neonates showed lower NET formation in

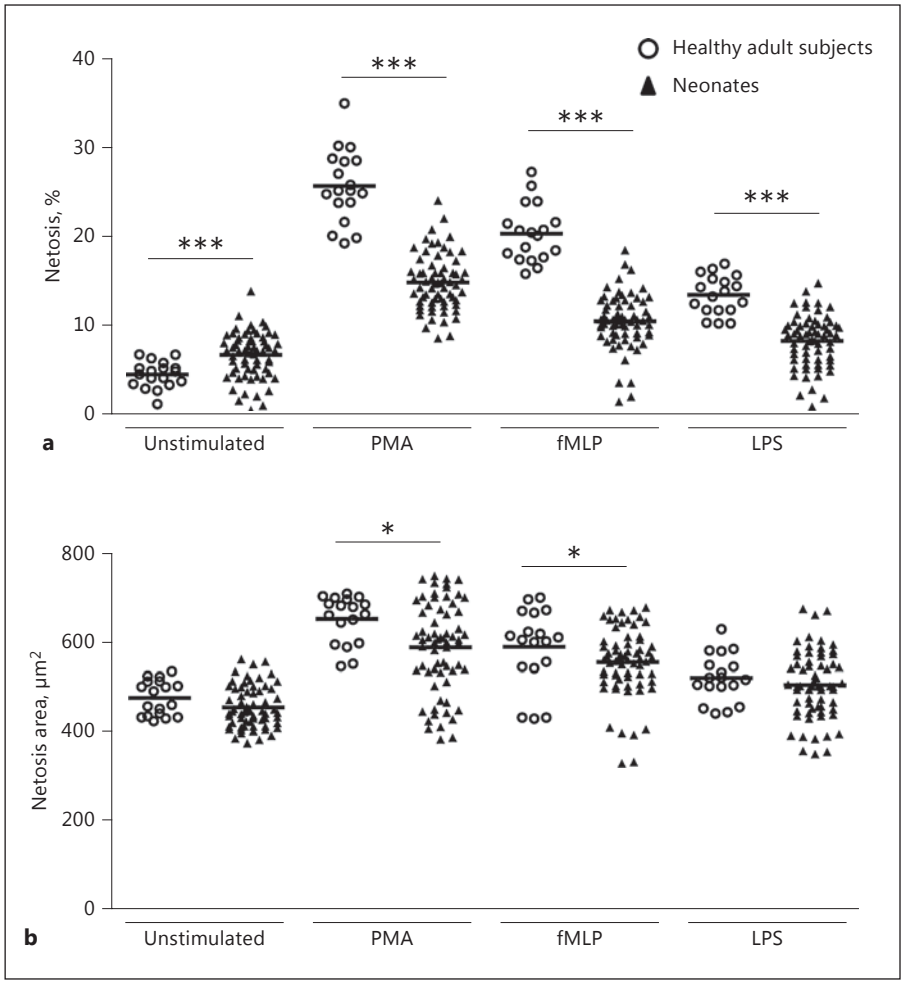

Fig. 2. NET-forming neutrophils of all neonates (n = 66) compared to healthy adult subjects (n = 18) in terms of mean percentage of NET-forming cells (**a**) and mean NET area (**b**). NET percentage significantly differed between groups following stimulation with phorbol 12-myristate 13-acetate (PMA), N-formylmethionine-leucyl-phenylalanine (fMLP), lipopolysaccharide (LPS) ($p < 0.0001$ each), and in the unstimulated controls ($p = 0.0007$). NET area significantly differed between groups following stimulation with PMA ($p = 0.0166$) and fMLP ($p = 0.0484$). * $p < 0.05$; *** $p < 0.001$.

terms of percentage and area. All neonates analyzed showed impaired NET formation upon stimulation with PMA, fMLP, and LPS ($p < 0.0001$ each) (Fig. 2a). The lower NET formation in neonates compared to adults was of a higher significance in terms of NET percentage than NET area. Only a tendency towards a difference in NET area was detected between the LPS group and the unstimulated controls (Fig. 2b).

PMA-induced NET formation was significantly lower in preterm infants than in term infants with regard to both NET percentage (Fig. 3a; $p = 0.0031$) and NET area (Fig. 3b; $p = 0.0313$). PMA-induced NET formation was also significantly lower in neonates with a low birth weight (<2,500 g) than in those with normal birth weight (≥2,500 g) in terms of both NET percentage (Fig. 3c; $p = 0.0014$) and NET area (Fig. 3d; $p = 0.0112$). Gestational age and birth weight were not associated with any significant differences in NET formation in the groups stimulated with fMLP or LPS, or in the unstimulated control group (data not shown).

Univariate analysis did not reveal any influence of perinatal infections, gender, or mode of delivery with one exception. We found that fMLP-induced NET formation was significantly lower in neonates delivered by secondary cesarean section than by spontaneous delivery (11.89 ± 3.00 vs. 9.36 ± 3.69%, p = 0.0184) (data not shown).

Discussion

Neutrophils play a major role in the innate immune systems, and newborns show significantly impaired neutrophil function [12], especially with regard to chemotaxis, pathogen recognition, and phagocytosis. Our results clearly demonstrated that neonates showed less formation of NET than adult controls. In controls, per-

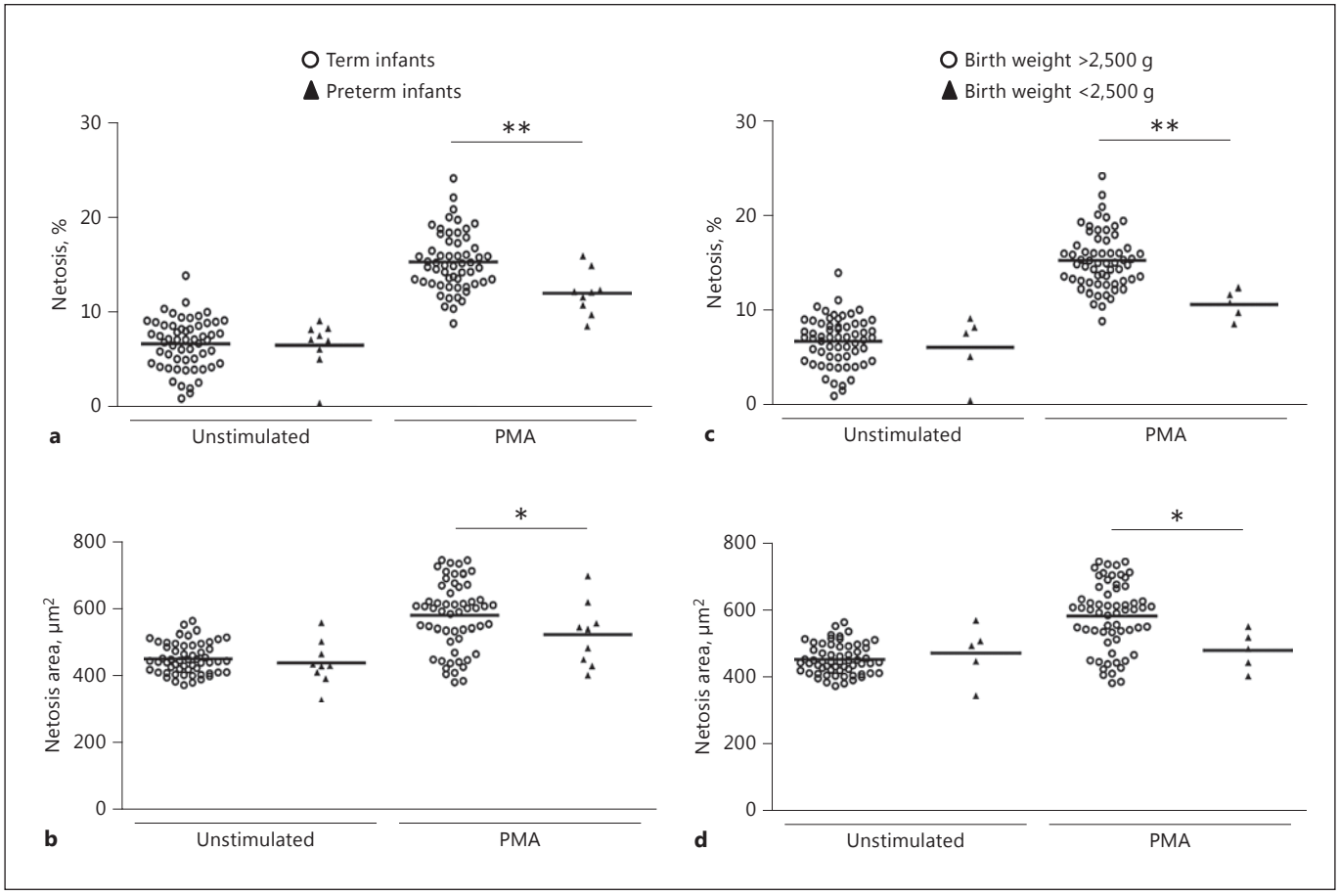

Fig. 3. Influence of gestational age and birth weight on NET formation. Term infants ($n = 57$) were compared to preterm neonates ($n = 9$) in terms of NET percentage (**a**) and NET area (**b**) following stimulation with phorbol 12-myristate 13-acetate (PMA). Additionally, infants with normal birth weight (≥2,500 g, $n = 61$) were compared to those with low birth weight (<2,500 g, $n = 5$) with regard to the percentage (**c**) and area (**d**) of NET-forming cells. Data are presented as means. * $p < 0.05$; ** $p < 0.01$.

centages of NET formation were comparable to those of earlier published data of our [9] as well as another group [20].

Our present data extend this previous knowledge of neonatal NET formation, revealing that neonatal neutrophils show NET production after a 2-h incubation with different stimuli. In contrast to these data, Yost et al. [16] reported that neonates could not form NETs 1 h after stimulation with PMA or LPS. Their study included particularly preterm infants ($n = 16$, 23–30 weeks of gestation), while our present study included neutrophils from a large number of term infants treated with LPS, PMA, and fMLP. These contradictory observations could be explained by differences in incubation time, gestational age, and stimuli. With a longer incubation time (3 h) of LPS stimulation, neonatal neutrophils were equally potent in NET formation as adult neutrophils [17]. However, this study consisted only of 3 neonates without specification of their gestational age, which is one of the most important factors in neonatology. Our data suggest that NET formation depends on maturation, because neonates showed less NET formation than an adult control group, and NET formation was further reduced in our subgroup of late preterm infants.

The present study considered only NET formation and not the effectiveness of NETS with regard to killing pathogens or any other functions. Due to the limited amounts of venous umbilical cord blood available, we restricted our study to NET analysis. Yost et al. [16] incubated neutrophils for only 1 h for NET formation and reported im-

paired bacterial killing. In contrast, Marcos et al. [17] found delayed but functional bacterial killing after a 3-h incubation. Recently, Byrd et al. [21] studied NET formation in healthy term neonates, demonstrating that neonatal neutrophils were capable of fungus-induced NET formation within 30 min, although neonatal neutrophils failed to build NETs after a 1-h stimulation with PMA or LPS. These data suggest that neonatal NET formation depends also on the type of stimulation. Although we did not study bacterial killing, we used fMLP as a stimulus, which mimics the N-formyl oligopeptides released by bacteria and activates circulating blood leukocytes by binding to specific G protein-coupled receptors on these cells [22]. We also used LPS, which simulates pathological stimulation by signaling via TLR2/TLR4 [23].

Another important factor influencing neonatology is the mode of delivery that might affect NET formation. Labor deliveries increase the surface expression of TLR on monocytes, and interleukin 8-induced neutrophil chemotaxis is enhanced due to the stress of birth [24]. In our cohort, the mode of delivery had no overall effect on NET formation.

Strengths of this study include the precisely timed blood collection from a large cohort of newborns. Additionally, we studied the effects of 3 different NET formation stimuli – PMA, LPS, and fMLP – and we observed a wide range of potentially relevant clinical factors. Since our results showed only a tendency towards a difference in association with gestational age and birth weight, further studies are required in a larger cohort – particularly a study including more immature infants. Moreover, the exact connection between NET formation and infection has yet to be determined [25]. Our study included a small number of neonates with perinatal infection, but these infants showed low illness severity and negative blood cultures. It is likely that NET formation is affected by prematurity, sepsis, and stress, since neonates reportedly show impaired oxidative burst [11]. Further research should include additional quantitation of NET formation not based solely on imaging, which is a limitation of our study. The recently described DNA/MPO ELISA assay is able to distinguish between NET formation and necrosis and is suitable for this purpose [26].

NETs are involved in T-cell priming and could also potentially be responsible for the activation of other cell types during infections [27]. Future studies should investigate this possibility, as well as how any relevant findings may impact neonates. NET production reportedly has harmful side effects related to several different diseases, including gout [25], thrombosis [28], and systemic lupus erythematosus [29].

Further investigation is required to elucidate the molecular principles behind impaired NET formation in neonates. Future studies may help to develop some sort of treatment option for this component of neonatal immune deficiency.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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